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PIG INTESTINAL PROLIDASE

**Purification, specificity, enzymatic and
structural properties**

Lund 1974

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structural properties**

by

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THIS DISSERTATION IS BASED ON THE FOLLOWING PUBLICATIONS:

- I. Purification and specificity of pig intestinal prolidase.
(Together with O. Norén and L. Josefsson)
Biochim. Biophys. Acta (1973) 327, 457-470.
- II. Enzymatic properties of pig intestinal prolidase.
Acta Chem. Scand. Submitted for publication.
- III. Structural properties of pig intestinal prolidase.
(Together with O. Norén)
Biochim. Biophys. Acta (1974) Accepted for publication.

In the text, the publications are referred to by their Roman numerals. The dissertation is also based on some unpublished results obtained by methods described in the above publications.



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NOMENCLATURE AND ABBREVIATIONS

The classification of proteolytic enzymes was generally accepted in the middle of the 1930s, and was stated by Bergman ¹.

Exopeptidase: A proteolytic enzyme, whose activity is restricted to terminal peptide linkages.

Endopeptidase: A proteolytic enzyme, whose activity is not restricted to terminal peptide linkages.

Aminopeptidase: An exopeptidase, whose activity is restricted to the aminoterminal peptide linkage.

Carboxypeptidase: An exopeptidase, whose activity is restricted to the carboxyterminal peptide linkage.

Dipeptidase: An exopeptidase, whose activity is restricted to the dipeptide linkage.

Prolidase: An exopeptidase, whose activity is adapted to the aminoacyl-proline peptide linkage.

Unless otherwise stated, the amino acids are of the L-configuration. In tables, the three letter abbreviations ² are used. The special peptide linkage created when proline or derivatives of proline take part with its nitrogen is called the 'aminoacyl-proline peptide bond'.

TNBS 2,4,6,-trinitrobenzenesulphonic acid

Cbz carbobenzoxy

DFP di-isopropylfluorophosphate

PCMB p-chloromercuribenzote

PHMB p-hydroxymercuribenzoate

CTP synthetase UTP:ammonia ligase (ADP), EC 6.3.4.2

S.D. standard deviation

S.E. standard error

K_m Michaelis-Menten's constant

K_i the inhibition constant

$s_{20,w}$ sedimentation coefficient corrected for water as solvent at 20°C and extrapolated to zero concentration

S Svedberg unit

$E_{1\%}^{1cm}$ The absorbance of a 1% solution through a path length of 1 cm.

INTRODUCTION

E a r l y d e v e l o p m e n t

The knowledge of proteolytic enzymes and proteins increased parallely during the 19th and early 20th century. During the latter part of this period, these enzymes also were a great aid in the understanding of enzyme mechanisms. The application of proteolytic principles to the digestion of proteins resulted in peptides called peptones and albuminoses. Studies of the trypsin and pepsin digestion of proteins led to the formulation of a digestion scheme in which proteins were believed to be absorbed as peptides (for ref., see 3 and 4). The discovery by Cohnheim ⁵ that a principle extracted from dog intestines digested pepton into amino acids was therefore of great importance for both the understanding of protein composition and digestion. He called the principle erepsin, I break.

T h e c o n c e p t o f d i p e p t i d a s e

The work of Fischer and collaborators at the turn of the century made available methods for the synthesis of simple peptides. It was soon demonstrated that some of them could be digested by a pancreas preparation ⁶ and by erepsin ⁷⁻⁹. Up to 1928, erepsin was considered an enzymatic entity, and the digestion of some synthetic dipeptides was studied ¹⁰⁻¹². v Euler and Josephson ¹³ showed that acylation of the free amino group of glycyl-glycine prevented hydrolysis. They postulated ¹⁴ the

combination of the amino group of the substrate with the enzyme.

The term dipeptidase was introduced in 1928 by Grassmann and Dyckerhoff¹⁵ who demonstrated that yeast autolysate was separated into a dipeptide and a polypeptide splitting component by adsorption on iron hydroxide. The dipeptidase preparation did not hydrolyze acylated dipeptides, polypeptides, or amino acid amides, which suggested that the dipeptidase required both a free amino and a free carboxyl group adjacent to the linkage to be split. It was later demonstrated that also pig erepsin could be resolved into the two components¹⁶. The specificity requirements of the dipeptidase, at that time considered a homogeneous enzyme, were later stated in more details¹⁷. As late as 1944, Smith and Bergmann¹⁸ questioned the existence of dipeptidases as a separate group of enzymes.

In 1929, Linderstrøm-Lang isolated another activity, leucyl peptidase, from erepsin hydrolyzing leucyl-glycine and leucyl-glycyl-glycine¹⁹⁻²¹. At the same time, it was suggested that an enzyme activity, prolinase²², was distinct from the intestinal aminopolypeptidase. It could split prolyl-glycin and prolyl-glycyl-glycin. Gailey and Johnson²³ suggested that erepsin was composed of a number of dipeptidases, on the basis of variation in activation behaviour.

The fundamental work on the dipeptidases presented by Smith and collaborators²⁴⁻²⁷ substantiated the heterogeneity of the dipeptidases and resulted in the classification of dipeptidases still in use. Partial purification followed by experiments on substrates with blocked amino and/or carboxyl groups confirmed a group of enzymes specific for dipeptides.

The first mammalian dipeptidase obtained in homogeneous form was a pig renal dipeptidase²⁸. Later the glycyl-leucine dipeptidase^{29, 30} and the prolidase³¹ from pig intestine and the glycyl-leucine dipeptidase from monkey intestine³² were purified and their specificity established.

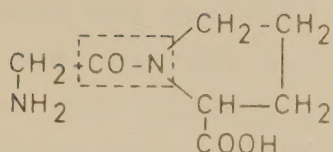
D i p e p t i d a s e s o u r c e s

Intestinal mucosa was the original source of erepsin and dipeptidase preparations. Preparations of yeast also resulted in substantial information rather early ^{10,15}. Around 1930, investigations began on dipeptidases from other sources, including bacteria and plants (for ref., see 33). In mammals, the dipeptidases have been demonstrated in almost every tissue studied (for ref., see 24, 25, 27, 34) and it was shown that some dipeptidase activities from four different pig tissues had accordant enzymatic properties ³⁵.

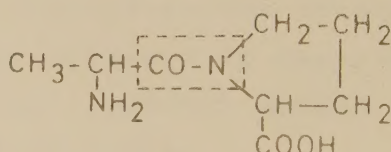
PREVIOUS INVESTIGATIONS ON PROLIDASE

Definition

Fischer and Abderhalden ³⁶ showed that the digestion of different proteins by a pancreas preparation resulted in an indigestible residue, which at chemical hydrolysis liberated large amounts of proline and phenylalanine. At the same time, they began a series of experiments for the synthesis of proline peptides. A report on the synthesis of leucyl-proline appeared in 1904 ³⁷, but some years later, it was shown to be premature ³⁸. It was not until 1931 that dipeptides of the general constitu-



GLYCYL-PROLINE



ALANYL-PROLINE

Fig. 1. The chemical constitution of glycyl-proline and alanyl-proline, indicating the characteristic of the aminoacyl-proline peptide bond.

tion aminoacyl-proline were synthesized and that the digestion of glycyl-proline by an erepsin preparation could be demonstrated ³⁹.

The carbobenzoxy method for the synthesis of peptides inaugurated a new era for the investigation of the dipeptidases. The synthesis of glycyl-prolin and alanyl-proline by this method was described in 1932 ⁴⁰. Glycyl-proline was not hydrolyzed by a pancreas preparation but both peptides were cleaved by an erepsin preparation. The hydrolysis was not caused by the 'dipeptidase' ¹⁶ component, but could be by the 'aminopolypeptidase' ¹⁶ component. On the basis of the indifference of the glycyl-proline hydrolysis to cyanide, a known dipeptidase and aminopolypeptidase inhibitor, it was suggested in 1936 that this activity was caused by a special enzyme ⁴¹.

This enzyme was called prolidase by Bergmann and Fruton in 1937 ⁴², and this name has since then been preferred. By prolidase was meant one of the exopeptidases whose activity was adapted to split the aminoacyl-proline peptide bond. This peptide bond differs from the usual peptide bonds, as its nitrogen lacks a bound hydrogen (Fig. 1), and has been called an imido peptide bond. The action of prolidase was thought to be confined to dipeptides; the enzyme was consequently classified as an 'imidodipeptidase'. Table I is a compilation of studies of the hydrolysis of prolidase substrates.

P u r i f i c a t i o n e x p e r i m e n t s

Smith and collaborators purified prolidase from pig intestine ¹⁸, horse erythrocytes ⁴³, and pig kidney ^{44,45}. The methods used included organic solvent-, salt-, and pH-fractionations.

Only the pig kidney preparation ⁴⁵ resulted in a considerable purification, partially because of the stabilization of the activity with Mn^{2+} and glutathione. A purification factor of 12 000 times was reported, and the best preparations were

TABLE I

A COMPILATION OF STUDIES OF THE HYDROLYSIS OF DIPEPTIDES BY CRUDE EXTRACTS AND PARTIALLY PURIFIED PROLIDASE PREPARATIONS FROM MAMMALS

Dipeptide	Reference number
Ala-Pro	31, 35, 40, 73-76, 83, 84, 86, 88, 89, 104, 105, 145-147, 149
Gln-Pro	47, 48
Glu-Pro	66, 77, 83, 104, 105, 145
Gly-Pro	18, 39-48, 52-55, 71, 72, 82, 139-144
Leu-Pro	46, 50, 64-67, 148
Phe-Pro	49, 50, 66, 71
Pro-Pro	43
Val-Pro	77, 83, 104, 145
Gly-Hyp	18, 43, 45, 46, 52, 53, 142
Phe-Hyp	43, 45
Pro-Hyp	43
Glycyl-D,L-proline	42
Glycyl-sarcosine	42, 45, 53
β -alaninyl-proline	43, 45, 54
Glycylallohydroxy-proline	45, 53
Glycyl-4-methoxy-proline	45, 53
Dehydrophenylalanyl-proline	43, 45

claimed to be 90% pure. Column starch electrophoresis resulted in one active peak, but the disagreement between activity and protein did not indicate that high degree of purity. The preparation was used in sequence work (see below) but no further characterization appeared. Smith concluded in his review on dipeptidases in 1960: 'As yet, none of these enzymes has been obtained in homogeneous form, and the specificity attributed to some of them must be regarded as only tentatively established.' 27.

More recently, Schwabe ⁴⁶ separated leucyl-proline hydrolyzing activity of bovine fibroblasts into two peaks, but he did not investigate it further. Rubino et al. ^{47, 48} were unable to separate human intestinal activities against glycyl-proline and glutaminyl-proline with established fractionation methods. Donlon and Fottrell ⁴⁹ reported activity against phenylalanyl-proline in their α -peptidase preparation, but could not demon-

strate any glycyl-proline or leucyl-proline activity. It is an interesting and puzzling finding in conflict with their earlier reports ⁵⁰.

S p e c i f i c i t y

The specificity of prolidase, as it was earlier known, was founded on the experiments of Smith and collaborators. Curiously, the purifications revealed little more about the specificity of this enzyme than was known from studies of much cruder preparations ⁵¹.

Prolidase was defined as a dipeptidase, because both free amino and free carboxyl groups adjacent to the sensitive bond were required. The enzyme had no activity against glycyl-glycyl-proline ^{43, 45}, glycyl-glycyl-hydroxyproline ⁴³, glycyl-prolyl-glycine ^{43, 45}, Cbz-glycyl-proline ^{42,43,45,52}, or Cbz-glycyl-hydroxyproline ^{45, 52}. The preparations from pig kidney and horse erythrocytes displayed no activity on peptides with the usual peptide bond ^{43, 45}.

Prolidase was found to be stereospecific, as only 50% of the racemic compound glycyl-D,L-proline was hydrolyzed ⁴². The substitution of proline with hydroxyproline in glycyl-proline and prolyl-proline caused a ten times slower reaction ^{18, 43, 45, 52, 53}. β -alanyl-proline was slowly split ^{43, 45, 54}, and dehydrophenylalanyl-proline was completely resistant to hydrolysis ^{43, 45}. Glycyl-sarcosine was shown to be hydrolyzed at a rate of about 10% of that of glycyl-proline ^{45, 53}, indicating that the pyrrolidone ring was an important feature of the most sensitive substrates of prolidase.

In the 1950s prolidase played a role in the discussion on the mechanism behind enzyme action ⁵¹. Fixed alterations of the radicals in the pyrrolidone ring caused different hydrolysis rates, which suggested steric effects on the interaction of the substrate with the enzyme ⁵³.

The strict dipeptidase nature of prolidase was later questioned ⁵⁵, as it was demonstrated that a highly purified prolidase preparation was able to hydrolyze glycyl-prolyl-leucine. This fact was used in sequence studies ⁵⁶⁻⁵⁸. An enzyme, aminopeptidase-P, capable of removing an aminoterminal amino acid bound to proline has been isolated and purified from E.coli ^{59, 60}. It was suggested that this type of enzyme was present in pig kidney and was responsible ⁵⁹ for the hydrolysis of glycyl-prolyl-leucine ⁵⁵. In fact, enzyme activities of aminopeptidase-P type have been isolated and purified from pig kidney (X-prolyl-aminopeptidase ⁶¹, tripeptidase ⁶², aminopeptidase M ⁶³), but in the recent review on dipeptidases, Delange and Smith wrote: 'Indeed, the alleged presence of aminopeptidase P in swine kidney extract may be due to prolidase.' ⁶³.

Finally, in experiments where dipeptidases have been histochemically stained after electrophoresis on agarose or starch ⁶⁴, the use of aminoacyl-proline peptides always gave a distinct electrophoretical pattern. This was in contrast to other peptides and indicated the restricted specificity of prolidase ^{50, 64-67}.

E n z y m a t i c p r o p e r t i e s

A moderately purified preparation from horse erythrocytes was rather stable ⁴³, whereas the pig kidney preparation was most unstable in its purified form; this obviously hampered the further characterization ⁴⁵. The reported pH-optima for prolidase of different origins varied. Table II lists the various values reported.

It was suggested that the glycyl-proline hydrolysis by prolidase followed a first order reaction ⁴³. The first order rate constant was linear with enzyme concentration over a wide range ^{43, 45}. Recently, Rubino et al. ⁴⁷ reported values of K_m for the hydrolysis of glycyl-proline (1.92 - 2.10 mM) and glutamyl-proline (2.93 - 6.66 mM) by a crude preparation from human intestine. Similar values were obtained by the use of an

TABLE II

pH-OPTIMA FOR THE ENZYMIC HYDROLYSIS OF TYPICAL PROLIDASE SUBSTRATES BY CRUDE EXTRACTS AND PARTIALLY PURIFIED PROLIDASE PREPARATIONS FROM MAMMALS

Dipeptide	pH-optimum	Source	Ref.no.
Ala-Pro	7.0	Human foetal and adult intestine	74, 75
	7.0	Pig foetal and adult intestine	146
	7.0	Rat foetal and adult intestine	73, 76
	7.3	Pig trachea	35
	7.0	Pig uterine tube	35
	7.0	Pig uterus	35
Gln-Pro	6.3-7.3	Human foetal intestine	48
	6.3	Human adult intestine	47
Glu-Pro	7.2	Human adult intestine	77
Gly-Pro	7.3-7.4	Human foetal and adult intestine	47, 48, 71
	8.0	Guinea-pig intestine	72
	7.8-8.0	Horse erythrocytes	43
	8.0	Pig kidney	45
	7.5-8.2	Rabbit muscle	52
Gly-Hyp	8.0	Pig kidney	45
Phe-Pro	6.0	Human adult intestine	71
Val-Pro	6.7	Human adult intestine	77

intestinal preparation from human foetus ⁴⁸. The turnover number of pig kidney prolidase was thought to be 350 000 moles of glycyl-proline per 100 000 g of enzyme ⁴⁵.

Prolidase was product-inhibited by proline (75 mM) and also inhibited by phenylhydrazine ⁴², pyrophosphate, EDTA, fluoride, and citrate ⁴³.

Johnson and collaborators in the late 1930s discovered that metals were essential for many peptidase activities ⁶⁸⁻⁷⁰. Prolidase was found to be activated by Mn^{2+} ¹⁸; this was later confirmed in many reports ^{43,45-48,51,52,71,72}. The grade of activation was shown to be time dependent ⁵², and the association constant for the reaction of Mn^{2+} with prolidase was calculated at 2.1×10^4 ⁴³. No significant activation by various crude extracts was noted when alanyl-proline, glutamyl-proline, phenylalanyl-proline, or valyl-proline were the substrates (no preincubation) ^{71,73-77}. Nor was the hydrolysis of glutamyl-proline activated by Mn^{2+} , despite preincuba-

tion ^{47,48}. Finally, many of the metals showed inhibitory properties ^{18,43,45,46}.

S t r u c t u r a l p r o p e r t i e s

Some structural properties of the purified pig kidney pro-lidase were reported ⁴⁵. Experiments with PCMB and iodoacetamide showed that the enzyme contained sulfhydryl group(s) necessary for its enzymatic activity ⁴⁵. After ultracentrifugation, the dominant peak (75%) corresponded to an $s_{20,w}$ of 6.4 S and a molecular weight of 150 000 was suggested ⁴⁵. The electrophoretic mobility was determined at $-5.2 \pm 0.5 \times 10^{-5}$ cm²/V sec ⁴⁵ and was found to decrease after the addition of Mn²⁺ ⁵¹. The enzyme displayed an absorbance spectrum of a typical protein ⁴⁴, and an analysis of the products after hydrolysis by paper chromatography revealed all common amino acids ⁵¹.

Schwabe ⁴⁶, using gel filtration on Biogel, found that the molecular weight of the bovine fibroblast activity against leucyl-proline was 150 000.

BIOLOGICAL ASPECTS

F u n c t i o n o f d i p e p t i d a s e s

The wide tissue distribution of the dipeptidases, which also applies to prolidase (Table III) raised doubts about the specific digestive function of these enzymes in the intestine ⁵². Coffey and de Duve ⁷⁸ showed that a liver lysosomal preparation digested proteins to dipeptides and amino acids, but that the peptidases of the cytosol resulted in further digestion. These results indicated a more general role of the dipeptidases in the intracellular protein catabolism.

TABLE III

DISTRIBUTION OF PROLIDASE ACTIVITY WITHIN VARIOUS
MAMMALIAN TISSUES

Tissue	Ref.no.
Mucosa of small intestine	39
Mucosa of trachea	35
Mucosa of uterine tube	35
Striated muscle	52
Heart muscle	52
Uterine muscle	52
Kidney	45
Fibroblasts (dental pulp)	46
Skin	139
Erythrocytes	43
Serum	141
Pituitary gland	142
Thymus	140

On the other hand, a variation of the cellular content of dipeptidase activities along the small intestine has been found ^{74,79-83}, which may reflect the different demands on peptide digestion along the intestine. This fact and also the high activity of the small intestine and low activity of the stomach and large intestine ⁷³⁻⁷⁵, suggest an adaptation of the dipeptidases to the digestion of intra-intestinal peptides, although a dual function of the intestinal dipeptidases cannot be excluded.

L o c a l i z a t i o n o f p r o l i d a s e

Nordström et al. ⁸⁴ demonstrated more activity in the villi than in the crypts of rat small intestine. As with the other dipeptidases (for ref., see 85), most of the prolidase activity has been found in the cell supernatant ^{67,72,86-88}. However, Heizer and Laster ⁷¹ claimed that some of the activity on glycyl-proline was localized in a particulate fraction of human intestinal mucosa. Josefsson et al. ⁸⁹ found more activity against alanyl-proline than against alanyl-glutamic acid or glycyl-leucine in the intestinal juice. No definite conclusion about the functional localization of prolidase has been reached, and it has been suggested that the dipeptidases in general act everywhere from the lumen of the intestine ⁹⁰, via the intestinal mucosa membrane ⁹¹, to the cytoplasm of the enterocyte ⁹².

It was recently demonstrated that certain dipeptides can be absorbed more rapidly than equimolar concentrations of the corresponding amino acids (for ref., see 93). Because most dipeptides are rapidly hydrolyzed, it was difficult to investigate the connexion between the dipeptidases and the carrier system. But glycyl-sarcosine--not much hydrolyzed by the intestine--was shown to accumulate in the enterocyte ⁹⁴, which indicated a transport of the dipeptide previous to hydrolysis.

F u n c t i o n o f p r o l i d a s e

None of the endopeptidases of the digestive tract can hydrolyze the aminoacyl-proline peptide bond ⁹⁵. Nor has any aminopeptidase capable of splitting this bond been described in the intestine; carboxypeptidase A was reported to have only an extremely weak action on Cbz-tryptophyl-proline ²⁵. However, Smith and Bergmann ¹⁸ reported hydrolysis of Cbz-glycyl-proline, Cbz-glycyl-hydroxyproline, Cbz-glycyl-prolineamide, Cbz-glycyl-hydroxyprolineamide and Cbz-prolyl-proline by an intestinal preparation. Wojanrowska and Gray ⁹⁶ separated and characterized three intestinal brush border peptidases, none of which showed activity against proline-containing peptides.

Generally, in living material, the enzymatic ability to hydrolyze the aminoacyl-proline peptide bond in oligopeptides seems to be restricted. Enzymes from kidney with activities on N-terminal aminoacyl-proline peptide bonds ⁶¹⁻⁶³ (cf. p. 12), were described whereas the activities of enzymes from spleen ^{97,98} and kidney ¹⁸ that were capable of removing a C-terminal proline have been reported; there are also solitary reports on aminoacyl-proline peptide bond hydrolyzing activities of microbial ⁹⁹ and plant ¹⁰⁰ origin.

Frazer ¹⁰¹ suggested that '...faulty handling of certain peptides in wheat gluten during their absorption...' was the major fault in coeliac disease. It was also shown that the toxic fraction of gluten, gliadine ¹⁰², was especially rich in glutamine (43%) and proline (12%) ¹⁰³. No specific enzyme deficiency, as analysed on biopsy material, however, has been detected ^{65,104-106}.

To summarize: the presence of prolidase seems to be of outstanding importance for the complete digestion of proteins in the gastrointestinal tract, whereas peptides including the usual peptide bond can be hydrolyzed by many peptidases with broad specificity. On this basis, a deficiency of prolidase will probably result in more pronounced biological effects than will a deficiency of the other peptidases.

PURPOSE OF THE INVESTIGATION

In a study of the processes of the final digestion and absorption of proteins, the fate of the aminoacyl-proline peptides were thought to be especially interesting, as it was known that a restricted number of peptidases can hydrolyze these peptides. With increased knowledge of these peptidases, it would be possible to investigate the detailed processes of the digestion absorption system handling this type of peptides.

The simple hydrolytic reaction and the possibility of small defined variations in the substrate for many years placed the dipeptidases in an advanced position in the study of enzyme mechanisms. Probably because of the lack of convenient purification methods, today's knowledge about the mechanism of dipeptide hydrolysis by dipeptidases is scanty, and other enzymes have come into the prominence as models in the discussion on enzyme mechanistics ¹⁰⁷.

These factors justified a closer study of prolidase. To obtain satisfactory information on the enzyme called for a convenient and reproducible purification method. This method should permit the preparation of amounts sufficient not only for the specificity and enzymatic studies, but also for the structural characterization. Organic solvents, earlier used in the fractionation of dipeptidases, were avoided, because it had been suggested that they induced specificity changes ^{108,109}.

PRESENT INVESTIGATION

A s s a y m e t h o d

Lindberg et al. ¹¹⁰ gave a review of peptidase assay methods. A simple and sensitive assay method suitable for scanning chromatographies was earlier developed in the laboratory ¹¹¹. The absorbance of the peptide bond in the low ultraviolet range was utilized, disturbing substances being precipitated with spectrophotometrically pure alcohol. Instead of glycyl-proline, alanyl-proline was chosen as substrate, as it is 3-4 times more sensitive to hydrolysis; thereby, the sensitivity of the prolidase assay was increased 3-4 times.

S o u r c e a n d c o l l e c t i o n o f e n z y m e

The choice of organ was obvious in view of our interest in the intestinal digestion. The small intestines were obtained from pigs, as this animal nutritionally resembles man ³⁴. Moreover, it was easy to obtain large amounts of pig intestines.

Josefsson and Sjöström's study ⁸⁶ formed the basis of a simple enzyme collection procedure with high capacity. Whole pieces of intestines were shown to liberate their dipeptidases into the extraction medium. The finding was not new; it was used in early investigations on erepsin ⁹. Through the years, however, most authors included a mucosal preparation by mechanically scraping the intestine and destroyed the cell by grinding with sand and later by homogenization.

An investigation into the influence of extraction time, and type and amount of extraction medium, showed that pieces of intestines liberated most of the prolidase activity into water in one hour. The extraction in water provided the basis for the subsequent lyophilization; a procedure that caused no loss of prolidase activity. The first two metres of the small intestine was discarded because of the possible presence of pancreatic proteolytic enzymes, the next three metres being used. With an extraction performed under optimum conditions, this preparation procedure, besides its simplicity, resulted in a circa threefold purification compared with a homogenate of scraped intestinal mucosa.

During the first part of this investigation (up to 1969) the intestines came from Scanian swine (Scan, Kävlinge, Sweden); in the later part, Danish swine were used. These intestines were delivered by Forenede Sjællandske Andelsslagterier, Roskilde, Denmark, and with the intestinal preparation 'Roskilde III' described in 1929 by Linderstrøm-Lang ¹⁹ in mind, we called our first Danish preparation 'Roskilde IV'.

P u r i f i c a t i o n (I)

A purification factor of 150-300 times was obtained by a process involving pH-fractionation, ammonium sulphate fractionation, DEAE-cellulose chromatography, and gel filtration on Sephadex G-200. The final purification was accomplished either by preparative polyacrylamide gel electrophoresis (Procedure I) or by a process utilizing the ability of the enzyme to aggregate to polymers (Procedure II). Table IV gives the purification results.

The initial pH-fractionation probably removed (a) substance(s) disturbing the binding of prolidase to a DEAE-ion exchanger. The lowest pH (5.2) still acceptable for stability reasons was chosen. As judged by the specific activity, the optimum fractionation time was two days. The ammonium sul-

TABLE IV

PURIFICATION OF PROLIDASE FROM PIG INTESTINAL MUCOSA

Figures are given for 100 g of intestinal powder.

Fraction	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)	Purification factor
Intestinal powder solution	28 200	0.305	100	1.0
Supernatant	20 600	0.266	73	0.87
(NH ₄) ₂ SO ₄ fraction	18 800	2.48	67	8.1
DEAE-cellulose fraction	15 900	26.2	56	86
Sephadex G-200 fraction	11 900	54.5	42	180
Purification Procedure I x)				
polyacrylamide electrophoresis fraction	5 540		20	
prolidase Preparation I	2 130	186	7.6	610
Purification Procedure II x)				
aggregated fraction	3 090	72.5	11	240
prolidase Preparation II	2 460	223	8.7	730

x) The figures, related to 100 g of intestinal powder, are based on an experiment where 2.4 % of the Sephadex G-200 fraction were purified according to Procedure I and the rest according to Procedure II.

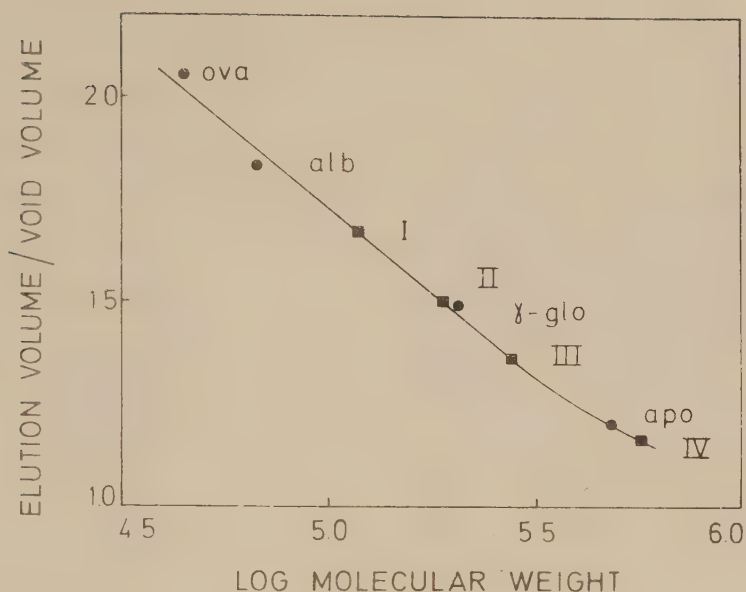
phate precipitation limits proved quite constant from one batch of enzyme to another, with no signs of more than one alanyl-proline hydrolyzing activity, as estimated by analytical experiments preceding each preparation. A thorough washing of the prolidase precipitate usually ensured a purification factor of about ten and a recovery of 70-80%.

The use of DEAE-cellulose offered the possibility of a chromatography with high load and excellent flow. When the enzyme solution was applied to the chromatographic column in the ratio of 200 g intestinal powder to 1 litre DEAE-cellulose and the column was eluted with a linear KCl gradient, the activity appeared as a symmetrical peak in a volume of 200-300 ml. The purification factor was about ten, and the recovery was good (80-90%). Analytical experiments had shown prolidase to be delayed by the use of chromatography either on Sephadex G-100 or Sephadex G-200. As judged by the purifi-

cation factor, the choice between them was not critical, but technical aspects gave preference to Sephadex G-100. However, in purification Procedure II, we considered Sephadex G-200 to be the best (see below). The gel filtration chromatography resulted in a purification of 2-4 times with an acceptable recovery (60-75%).

It was found that prolidase was simply and quantitatively eluted from the gels after polyacrylamide gel electrophoresis. This formed the basis for a preparative gel electrophoresis step. The gels of the standard analytical system of Davis ¹¹² and Ornstein ¹¹³ were magnified to a diameter of 1 cm. After electrophoresis, the gels were sliced and eluted. A homogeneous preparation resulted when as much as 1 mg was applied to each gel.

At a certain stage in the investigation, the prolidase appeared inhomogeneous concerning enzymatic activity. In chromatographies on Sephadex G-100, the activity appeared everywhere from void volume to the normal position. The use of isoelectric focusing resulted in wide, heterogeneous peaks; furthermore, prolidase was separated into several peaks by the step-wise elution of a DEAE-Sephadex column. The results of the gel filtration experiments indicated that the behaviour in the different systems depended on the appearance of polymeric forms. Fig. 2 gives the results of a molecular size determination of some of the forms separated by chromatography on Sephadex G-200. The results are consistent with molecular weights of 119 000, 190 000, 280 000 and 576 000, suggesting a monomer, dimer, trimer and hexamer. It has since been shown that these polymers were formed if the prolidase solution was highly concentrated. After treatment with 0.1 M mercaptoethanol, the activity again appeared homogenous in gel filtration experiments, with a molecular size corresponding to the original (Carlsen, Sjöström and Josefsson, to be published). As the spontaneous polymerization was prevented by the presence of mercaptoethanol and EDTA, these compounds were added to the buffers used during the purification process.



F i g . 2 . Molecular size determination of different fractions from a chromatography of the aggregated forms ¹⁵⁰. The experiment was run on a Sephadex G-200 superfine (column size 1.5 cm x 86.3 cm), equilibrated in and eluted with 0.05 M Tris-HCl buffer, pH 7.5. About 1 unit of activity of each fraction was applied. ova ovalbumin, alb albumin, γ-glo γ-globulin, apo apoferritin. I, II, III, IV different forms of prolidase.

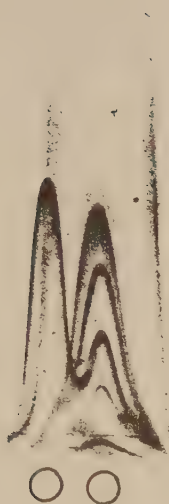
This ability to control the polymerization reaction made possible another final purification process. Generally, a total purification can be obtained if the property upon which a chromatography is based can be specifically changed between two identical runs. The methionine diagonal method of Tang and Hartley ¹¹⁴ exemplifies this. The change in molecular weight due to the polymerization, together with gel filtration, could thus be exploited. During the course of these experiments, this application on the purification of CTP synthetase was reported ¹¹⁵. However, for prolidase, the polymerization was difficult to achieve in impure preparations; furthermore, the aggregation was not specific. Therefore the pro-

cedure was used only for a final purification.

If the polymerization reaction was allowed to proceed long enough (weeks), we sometimes obtained total polymerization in analytical experiments (Carlsen, Sjöström and Josefsson, to be published). For preparative purposes, a rest of about 20% monomer was the rule. As the possibility of obtaining a pure product depended on the separation of species with a molecular weight equal to or higher than the prolidase monomer, chromatography on Sephadex G-200, instead of Sephadex G-100, was preferred. None the less, in the chromatography of the aggregated enzyme, some activity just up to the monomer had to be discarded. Moreover, the transformation of the monomer to polymer was accompanied by some loss of activity which was not regained in the reversed reaction, and finally, rechromatography steps on Sephadex G-200 sometimes were necessary; therefore the two chromatographies with polymer and monomer resulted in a rather low recovery. The product, however, was a pure prolidase.

The results in Table IV are representative of rather many preparations. Procedure I was suitable for preparing minor amounts for enzymatic and specificity studies. Procedure II resulted in 5-10 mg pure enzyme per 100 g of intestinal powder. Normal laboratory facilities could handle 200 g intestinal powder.

To exclude the possibility of bacterial origin of the purified prolidase, the following immunological comparison with a partly purified prolidase from pig uterine tube was performed (unpublished). After extraction of uterine tubes with water and a subsequent lyophilization, the sample was chromatographed on a DEAE-cellulose column with a linear increasing KCl gradient. The most active fractions were pooled and concentrated by ultrafiltration. The preparation obtained (uterine tube prolidase), was immunologically compared with the purified prolidase, according to the earlier-described principles 116-118. The result obtained (Fig. 3) showed a close immunological relation between the intestinal prolidase and one of the uterine tube proteins, and thus strongly suggested that prolidase was of



F i g . 3 . Immunoelectrophoresis of the uterine tube prolidase and the pure pig intestinal prolidase. Two wells (2 mm diameter) were punched. In the left well, 2 μ l of a prolidase Preparation II (74 units of activity/ml) was applied; in the right, 2 μ l of the uterine tube prolidase (32 units of activity/ml) was applied. After 2 h diffusion time, the electrophoresis was run at 3 V/cm for 17 h.

mammalian origin. The experiment also further demonstrated the similarity between prolidasases from different sources.

P u r i t y (I)

Purity was originally judged by three methods: polyacrylamide gel electrophoresis ^{112,113}, crossed immunoelectrophoresis ^{119,120}, and the correspondence between enzymatic activity and protein in the final purification steps. During the further characterization, more indications of purity accumulated.

When the purified prolidase was run on polyacrylamide gel electrophoresis, a series of bands appeared, all with lower mobilities than the main band. They were found irrespective of which of the two pure preparations was applied. For many rea-

sons, these bands were interpreted as the polymeric forms. The treatment of the enzyme with 0.1 M mercaptoethanol, the elution from the preparative gels into a mercaptoethanol holding buffer, and also the inclusion of mercaptoethanol in the buffer of the final purification step greatly diminished the intensity of these bands. Moreover, the gel electrophoretic analysis of different polymeric forms, separated by gel filtration on Sephadex G-200 (Fig. 2) displayed Coomassie Brilliant Blue positive bands in identical positions. The two pure preparations displayed homogeneity (except minor polymeric bands) applying up to about 10 μ g to a gel. This corresponded to a purity of at least 99%.

A rabbit polyvalent antiserum was raised by immunizing rabbits with the Sephadex G-200 fraction, and an immunoglobulin solution was prepared ¹²¹. Crossed immunoelectrophoresis was performed on the two pure preparations, and the results confirmed the purity of the enzyme. Finally, in the last purification step of each procedure, there was good agreement between protein and the prolidase activity.

The equivalence in specificity spectrum between the two preparations (see below) gave evidence of the absence of other peptidases. Furthermore, the gel electrophoresis in dodecyl sulphate ¹²² displayed only one band. This finding, together with the homogeneity in the ordinary polyacrylamide gel electrophoresis ^{112,113} was, strong evidence for purity, as it was improbable that two proteins, running together in the ordinary polyacrylamide gel electrophoresis ^{112,113}, would have equal mobilities in the gel electrophoresis in dodecyl sulphate ¹²². The amino acid composition, as determined on different preparations, showed good agreement, indicating the reproducibility of the purification procedure.

S p e c i f i c i t y (I)

In relation to earlier knowledge about prolidase specificity, four things remained:

1. to group prolidase within the exopeptidases (aminopeptidase, carboxypeptidase, or dipeptidase).
2. to confirm the proposed specificity against the aminoacyl-proline peptide bond.
3. to elucidate what influence the N-terminal amino acid had on the hydrolysis rate, the results of which would be of interest to compare with the intestinal absorption data of these peptides.
4. to investigate whether prolidase was responsible for all imido(di)peptidase activity in the intestine.

The main assay method was that of Josefsson and Lindberg ¹¹¹. By replacing ethanol with meta-phosphoric acid, the efficiency of the protein precipitation was somewhat increased, resulting in better sensitivity of the mucosal extract analysis ³⁰. For substrates not suitable for this assay method, the TNBS-method of Binkley ¹²³, as modified by Norén et al. ³⁰, was used. We used the automatic amino acid analysis ¹²⁴ for analysing the activity against glycyl-glycyl-proline and glycyl-prolyl-alanine. Acidification and deproteinizing of the sample and also interruption of the enzymatic reaction were all accomplished in one step by the addition of 0.2 M citrate buffer, pH 2.2. It proved to be almost as efficient a precipitating agent as alcohol. After centrifugation, the supernatant was analysed for liberated amino acids. This application has also been reported by Maroux et al. ¹²⁵. All incubations were performed at pH 7.4, the concentration being 1.8 mM for the aminoacyl-proline peptide bond substrates and 8.5 mM for the others. The incubation time was, for stability reasons, maximized to 60 minutes.

Table V gives the results of the specificity analysis.

ad 1. The purified prolidase proved to be a strict dipeptidase, as no activity was detected against the two tested tripeptides, although the sensitivity of our assay was 2-10 times higher than that of Hill and Schmidt ⁵⁵ when they questioned the dipeptidase nature of prolidase.

TABLE V

SUBSTRATE SPECIFICITY OF PIG INTESTINAL PROLIDASE IN RELATION TO MUCOSAL EXTRACT

Figures are given as a percentage of the activity against L-alanyl-L-proline. Zero activity in the table means an activity less than 0.1 % of the activity against L-alanyl-L-proline. Exceptions are only obtained in the figures for the activity of the Mucosal extract against L-prolyl-L-proline and glycyl-sarcosine where the assay method did not permit estimations less than 5 %.

Substrate	Activity		
	Mucosal extract	Prolidase Preparation I	Prolidase Preparation II
Ala-Pro	100	100	100
Gly-Pro	30	30	30
Met-Pro	40	50	40
Glu-Pro	40	30	30
Ser-Pro	90	80	90
Gly-Hyp	0.3	0.5	0.4
Pro-Pro	0	0	0
Gly-Gly-Pro	+	-	-
Gly-Pro-Ala	+	-	-
Gly-Sarc	0	3	1
Gly-Gly	60	0	0
Ala-Leu	700	0	0
Leu-Ala	500	4	7
Ala-Ser	4000	0.2	0.2
Ser-Ala	1000	1	2
Ala-Glu	1000	0	0
Glu-Ala	200	2	3
Ala-Lys	400	0	0.5
Lys-Ala	70	6	7
Gly-Gln	700	0.1	0
Gln-Gly	600	0.1	0.2
Pro-Ala	70	0.3	0.4
Ala-Tyr	200	0	0
Tyr-Ala	500	6	0.4
Ala-Trp	1000	0	0
Trp-Ala	100	2	0.8

(+ means activity, - means no activity, see text)

ad 2. A series of dipeptides, in which alanine--both as C- and N-terminal--was combined with representatives of different types of amino acids, were used. The results showed that prolidase displayed a low but significant activity against some peptides with a structure other than aminoacyl-proline. Alanyl-proline, however, was twenty times more sensitive to hydrolysis than, for instance, leucyl-alanine and lysyl-alanine. It is suggested that these activities are real activities of the prolidase, as the specificity spectra of the two preparations closely resembled each other.

ad 3. Among the aminoacyl-proline peptides, the variable amino acid was found to influence the hydrolysis rate rather considerably, from prolyl-proline, not hydrolyzed at all, to alanyl-proline and seryl-proline, being hydrolyzed most rapidly. Prolyl-proline was earlier reported to be hydrolyzed by the erythrocyte prolidase at a rate of 25% of that of the hydrolysis of glycyl-proline ⁴³.

ad 4. A mucosal extract was also investigated for its dipeptidase activity to search for evidence of more than one imido-dipeptidase activity. The figures of hydrolysis for the different aminoacyl-proline dipeptides, however, agreed well between the two purified preparations, on the one side, and the mucosal extract, on the other, suggesting that only one molecular species, prolidase, accounts for all imidodipeptidase activity in the intestinal mucosa.

The mucosal extract was also tested for activity on the two tripeptides to get evidence of possible imidopeptidase activities of the small intestinal mucosa. It was found to split glycyl-glycyl-proline and glycyl-prolyl-alanine. When glycyl-glycyl-proline was incubated with a mucosal extract, the analysis of the reaction products showed, in addition to glycyl-proline, the presence of four times more glycine than proline. The rate of the proline release corresponded to 10% of the rate of the alanyl-proline hydrolysis. When glycyl-prolyl-alanine was incubated, all three amino acids were found to be liberated at a rate corresponding to 5-10% of the rate of

alanyl-proline hydrolysis (unpublished). The activity on glycyl-glycyl-proline can be ascribed to an amino(tri)peptidase, as suggested on intestinal ¹⁸ and muscle ⁵² preparations. Concerning the hydrolysis of glycyl-prolyl-alanine the reason might be carboxypeptidase A, the presence of an enzyme similar to aminopeptidase-P ^{59,60}, or a dipeptidyl peptidase ¹²⁶. Further studies will show which enzyme in the intestine can hydrolyze the aminoacyl-proline peptide bond in peptides greater than dipeptides.

E n z y m a t i c p r o p e r t i e s (I I)

The assay method ¹¹¹ was somewhat modified to make it suitable for enzymatic characterization studies. The ethanol precipitation procedure was omitted, as the pure enzyme exhibited negligible absorbance. In the fixed time version, the volumes of enzyme and buffered substrate solutions were increased to get more precise volumes. The enzymatic reaction was interrupted by the addition of 0.5 M o-phosphoric acid. A continuously recording version of the method was chosen for the determination of the kinetic coefficients. In this version, the enzyme solution volume was kept small (20 μ l) to obtain a rapid and complete mixing when the substrate solution (1000 μ l) was afterwards pipetted. The absorbance spectrum of the aminoacyl-proline peptide bond (Fig. 4) permitted variation of wavelength to compensate for variations in the substrate concentrations. Glycyl-proline was included as substrate because the earlier experience of prolidase was gained on this substrate.

The stability of prolidase was studied from three different aspects. The relative influence of pH on stability was studied to obtain a pH-optimum curve that reflected only the kinetics of the reaction. Information about how long the enzyme could be stored before incubation and how long the incubation could continue was also essential. Therefore the stability was studied at 0°C and 25°C with protein concentrations in parity

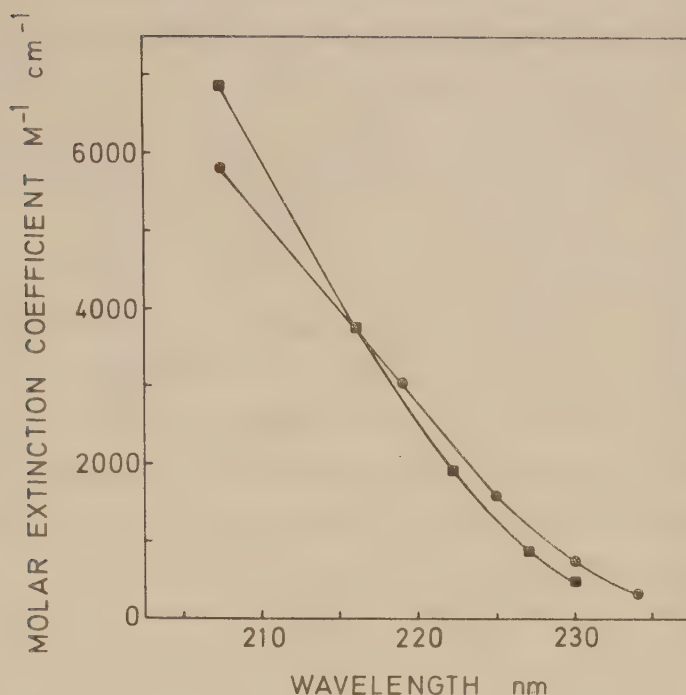


Fig. 4. The ultraviolet spectra of the aminoacyl-proline peptide bond in glycyl-proline and alanyl-proline. Appropriate concentrations of the peptides were measured with equimolar concentrations of the amino acids as reference. The compounds were dissolved in 0.1 M potassium phosphate buffer pH 7.4 (glycyl-proline) and 6.8 (alanyl-proline). ■ glycyl-proline, ● alanyl-proline.

with those before and during an assay.

We found no stability differences between pH 5.5 and 8.5. At 0°C and pH 6.8, a 10-20% loss of activity occurred during the first hour, followed by a continuous minor loss. Based on this result, the enzyme stock solution was diluted one hour before an assay and kept at 0°C. A similar instability at 25°C despite preincubation at 0°C was probably of minor importance, because progression curves that were run at different

enzyme concentrations showed identical profiles when hydrolysis percentage was plotted against the product of time and enzyme concentration, thus suggesting a substrate stabilization.

pH-optima were determined at 6.8 for the hydrolysis of alanyl-proline and 7.4 for the hydrolysis of glycyl-proline. The pH-optimum analyses were only performed in the pH-range, where pH was of no importance for the stability.

The Michaelis-Menten's constants for the reaction with glycyl-proline and alanyl-proline were found to be 0.13 mM and 0.29 mM, respectively by the use of Wilkinson's statistical estimation method ¹²⁷. It also permitted the calculation of the S.E. for the K_m -values (0.015 mM and 0.025 mM, respectively).

Of the amino acids tested, only proline was found to be an inhibitor of the enzyme. K_i for the inhibition reaction was found, by the above method, to be 0.49 mM at pH 6.8 and 0.21 mM at pH 7.4. The t-test ¹²⁸ in the comparisons of the kinetic coefficients determined with and without proline present suggested that proline was a competitive inhibitor. In an earlier report ⁴³, proline was found to be inhibitory only at rather high concentrations, which was consistent with the competitive behaviour, as that study ⁴³ used a substrate concentration of 50 mM.

Jennings and Niemann's equation ¹²⁹ for a product inhibited progression curve, inserted with the found kinetic coefficients, agreed well with the experimental curves. This knowledge of the course of the progression curves makes possible information from the whole curve for the calculation of the kinetic coefficients. This is much easier to handle than the repeated analyses on initial velocities. Moreover, it was possible to calculate the maximum velocity from assay data at every substrate concentration, without the limitation of having to measure at the initial part of the progression curve. Thus the molecular activities (3×10^4 moles of alanyl-proline $\times$ mole prolidase⁻¹ $\times$ min⁻¹, 8×10^3 moles of glycyl-proline $\times$ mole prolidase⁻¹ $\times$ min⁻¹) were determined from the assay values at 1.8 mM substrate concentration and a molar ex-

tion coefficient of $11.2 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ (III).

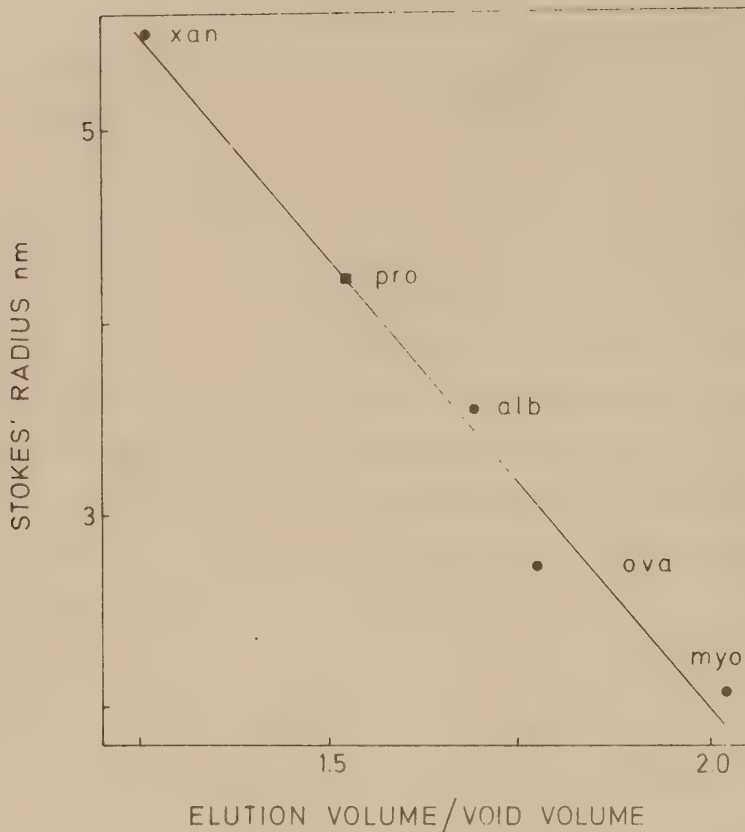
Ag^+ , Cu^{2+} , Hg^{2+} , and PHMB inhibited the enzyme and DFP did not; thus prolidase might be classified as a sulfhydryl enzyme.

The finding that thiol compounds and 1,10-phenantroline inhibited the enzyme indicated a metal content of prolidase. The inhibition with thiol compounds agreed with an earlier finding ⁴⁵ that glutathione inhibited prolidase. It can be explained by the binding of the sulphur to an essential metal of the enzyme. The partial reversibility of the 1,10-phenantroline inhibition with Zn^{2+} but not with Co^{2+} , Mg^{2+} , or Mn^{2+} suggested that zinc was involved in the catalysis. The earlier reported experiment ⁴⁵ on the dependence of prolidase activity on the Mn^{2+} concentration could be repeated, but the explanation of the result was a stabilization rather than an activation (unpublished). The presence of Co^{2+} , Mg^{2+} , Mn^{2+} , or Zn^{2+} at rather low concentrations (10 and 100 μM) during the hydrolysis did not influence the activity on glycyl-proline or alanyl-proline, a fact which together with the stability of the prolidase activity in the presence of 1 mM EDTA (I) indicated that the metal was strongly bound to the enzyme.

S t r u c t u r a l p r o p e r t i e s (I I I)

Stokes' radius of the purified prolidase was determined by gel filtration on Sephadex G-200. The values on the Stokes' radii of the protein standards were taken from Andrews ¹³⁰. Fig. 5 gives the result and corresponds to a Stokes' radius of 4.2 nm for prolidase. Assuming the same form and hydration of all proteins included in the experiment, a molecular weight of $108\,000 \pm 5000$ ($\pm$ S.D.) was calculated.

To obtain information about a possible subunit structure of prolidase, samples of it were treated with 2-mercaptoethanol and dodecyl sulphate. In some experiments, only dodecyl sulphate treatment was used. The resulting dodecyl sulphate-protein complexes were analysed regarding molecular weight,



F i g . 5 . A plot ¹⁵⁰ of elution volume/void volume v. Stokes' radii for a series of standard proteins in order to determine the Stokes' radius of prolidase. About 2 units of prolidase activity in 2 ml was applied to a Sephadex G-200 column (2.5 cm x 93.0 cm) equilibrated in and eluted with 0.05 M Tris-HCl buffer, pH 7.5. xan xanthine oxidase, pro prolidase, alb albumin, ova ovalbumin, myo myoglobin.

with polyacrylamide gel electrophoresis ¹²² and gel filtration on Sepharose 6 B ¹³¹, both performed in dodecyl sulphate. The molecular weight standards (albumin, ovalbumin, chymotrypsinogen, and myoglobin) were in all experiments treated with both mercaptethanol and dodecyl sulphate. Irrespective of the type of pretreatment, the molecular weight of prolidase was found to be 53 000 in both the gel electrophoresis and the gel filtration experiments. The results suggested that prolidase was

composed of two subunits with equal molecular weight and associated with each other by non-covalent forces, but further studies are required.

Isoelectric focusing found the isoelectric point of prolidase to be 4.4. The value agreed well with our determinations of the pI of prolidase in a crude extract, indicating that the preparation procedure had caused no great structural changes.

The amino acid composition was quantitatively analysed by automatic amino acid analysis ¹³². Cysteine and methionine were determined as cysteic acid and methionine sulphone ¹³³, respectively, whereas tryptophan was analysed spectrophotometrically ¹³⁴. The results (Table VI) showed that the protein

TABLE VI

AMINO ACID COMPOSITION OF PIG INTESTINAL PROLIDASE

Unless otherwise stated the values are the mean of three different analyses. The number of residues per molecule prolidase is calculated on the basis of a molecular weight of 108 000.

Amino acid	Mole %	Number of residues per molecule prolidase
Ala	6.64	65
Arg	6.34	62
Asx	7.92	77
Cys *	3.96	39
Glx	10.02	100
Gly	10.52	103
His	4.03	39
Ile **	3.94	38
Leu	7.86	77
Lys	3.78	37
Met ***	2.70	26
Phe	4.58	45
Pro	3.78	37
Ser *	5.87	57
Thr *	5.10	50
Trp **	1.27	12
Tyr	3.03	30
Val **	8.64	84
Total number of residues		978

* Determined as cysteic acid after performic acid oxidation ¹³³

** After 72 h hydrolysis

*** Determined as methionine sulphone after performic acid oxidation ¹³³

* Extrapolated to zero time by assuming a destruction according to a first order reaction

** Determined spectrophotometrically ¹³⁴

was especially rich in the neutral, aliphatic amino acids, glutamic acid/glutamine and aspartic acid/asparagine. The content of half-cystine was also relatively high. The precision of the amino acid analysis was quite acceptable, as the calculated standard deviations corresponded to one amino acid or less per molecule of prolidase for the stable and quantitatively liberated amino acids.

Based on the determined amino acid composition, the percentage content of leucine and arginine was calculated. With these factors, the total amount of protein in an analysed sample was calculated from the leucine or arginine content. Based on the absorbance of the sample, the $E_{1\text{ cm}}^{1\%}$ value was found to be 10.4. Assuming a molecular weight of 108 000 the molar extinction coefficient was computed to be $11.2 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$.

No N-terminal amino acid was found with the dansylation method ¹³⁵. In parallel experiments, the N-terminal amino acid of ribonuclease, lysozyme, and the soluble pig intestinal dipeptidase ³⁰ were detected. Three different reasons can be advanced to explain the negative result on prolidase; a blocked N-terminal amino acid, an N-terminal tryptophan, and an N-terminal amino acid unreactive with dansyl chloride for steric reasons.

The number of free sulfhydryl groups was analysed by a method utilizing Ellman's reagent ¹³⁶ and a method (Carlsen, J.B., to be published) taking advantage of the PHMB reaction with sulfhydryl groups of proteins ¹³⁷. Ellman's reagent resulted in 4-8 SH-groups per molecule of enzyme; PHMB gave somewhat higher figures (6-10). A greater reactivity with PHMB to the sulfhydryl groups of the enzyme was probably the reason. The variation in numbers of sulfhydryl groups could be explained by different content in the three different preparations analysed, as the results of analyses of one preparation were of smaller range. Thus this variation in sulfhydryl groups is real and can be explained by a long purification procedure, including changes in mercaptoethanol concentration.

The calculation of the number of disulphide bonds from the

amino acid analysis and the number of free sulfhydryl groups produced a figure of about 15. Prolidase was thus rather cross-linked with disulphide bridges, which would give it a somewhat compact and stable structure. This can explain the relative stability of prolidase during the purification process and also the observation that prolidase treated with 1% dodecyl sulphate at room temperature was still enzymatically active (unpublished). The compact molecule was also reflected in the poor reactivity of its SH-groups to Ellman's reagent, despite the addition of 0.5% dodecyl sulphate, and might explain the negative results in the experiments on the N-terminal amino acid.

Finally, prolidase was found to be a glycoprotein. The carbohydrate content was found to be low (about 0.5%) as estimated by the intensity of the Schiff periodic acid stain ¹³⁸ on polyacrylamide gels after performed electrophoresis. The finding is possibly an indication of a membrane origin of prolidase. Two other intestinal peptidases have recently been reported to contain carbohydrate ^{30,125}.

SUMMARY

Prolidase (imidodipeptidase, EC 3.4.3.7) is an exopeptidase, whose activity is adapted to split aminoacyl-proline peptide bonds. Since its definition in 1937, a few purification reports have appeared, but only a preparation from pig kidney resulted in a considerable purification. Prolidase was thought to be a dipeptidase. With glycyl-proline as substrate, the enzyme was early shown to be specifically activated by Mn^{2+} . A molecular weight of about 150 000 was suggested. The enzyme is widely distributed in living tissues, but little is known about its biological role. The high activity in the intestinal mucosa has, as for other dipeptidases, suggested a digestive function. It is the only known intestinal enzyme that digests aminoacyl-proline peptide bonds at a reasonable rate.

In this thesis, two alternative purification processes for prolidase were devised. After purification with pH-fractionation, ammonium sulphate fractionation, and chromatography on DEAE-cellulose and Sephadex G-200, the final purification was accomplished either by polyacrylamide gel electrophoresis or by a method utilizing the property of the enzyme to polymerize. The latter method allows the purification of 20 mg of prolidase each time. The enzyme had a specific activity of about 200 units of activity per mg protein when determined against alanyl-proline.

Prolidase was found to be more than 99% pure when tested on polyacrylamide gel electrophoresis, and proved to be homogeneous in crossed immunoelectrophoresis.

The specificity analysis showed that the enzyme was a specific dipeptidase. The activity was almost limited to dipeptides which included the aminoacyl-proline peptide bond. The influence of the N-terminal amino acid in the dipeptides on the reaction rate was studied. Prolidase was found to be responsible for all intestinal activity against the aminoacyl-proline and aminoacyl-hydroxyproline substrates.

The basic enzymatic properties of the enzyme were studied. pH-optima and K_m of the reactions with alanyl-proline (6.8 and 0.29 mM) and glycyl-proline (7.4 and 0.13 mM) and also the K_i of the found inhibition with proline (0.21 mM at pH 7.4 and 0.49 mM at pH 6.8) were determined. The values were used for the calculation of the theoretical progression curves, which agreed well with the experimental curves. Used as a stabilizer of the enzyme preparation, 2-mercaptoethanol showed inhibitory properties. The prolidase activity was not affected by DFP, whereas thiol-reagents inhibited the enzyme. Bivalent metal ions showed no significant influence on the enzyme activity. Inhibition with 1,10-phenantroline, partially reversible by Zn^{2+} , suggested this metal to be involved in the catalysis.

Some structural properties of prolidase were also determined. The molecular weight was found to be 108 000 under native conditions and 53 000 under denaturing conditions. The molar extinction coefficient ($11.2 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$), the isoelectric point (4.4), and the amino acid composition of the enzyme were determined. The number of free sulfhydryl groups per molecule of prolidase was estimated. The enzyme was shown to be a glycoprotein, containing about 0.5% carbohydrate. No N-terminal amino acid was found. The results suggested that the prolidase molecule was composed of two subunits with equal molecular weight, stabilized by about 15 disulphide bonds into a rather rigid conformation.

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